***Streptomyces scabies* 87-22 Contains a Coronafacic Acid-Like Biosynthetic Cluster That Contributes to Plant–Microbe Interactions**

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Plant-pathogenic *Streptomyces* spp. cause scab disease on economically important root and tuber crops, the most important of which is potato. Key virulence determinants produced by these species include the cellulose synthesis inhibitor, thaxtomin A, and the secreted Nec1 protein that is required for colonization of the plant host. Recently, the genome sequence of *Streptomyces scabies* 87-22 was completed, and a biosynthetic cluster was identified that is predicted to synthesize a novel compound similar to coronafacic acid (CFA), a component of the virulence-associated coronatine phytotoxin produced by the plant-pathogenic bacterium *Pseudomonas syringae*. Southern analysis indicated that the *cfa*-like cluster in *S. scabies* 87-22 is likely conserved in other strains of *S. scabies* but is absent from two other pathogenic streptomycetes, *S. turgidiscabies* and *S. acidiscabies*. Transcriptional analyses demonstrated that the cluster is expressed during plant–microbe interactions and that expression requires a transcriptional regulator embedded in the cluster as well as the *bldA* tRNA. A knock-out strain of the biosynthetic cluster displayed a reduced virulence phenotype on tobacco seedlings compared with the wild-type strain. Thus, the *cfa*-like biosynthetic cluster is a newly discovered locus in *S. scabies* that contributes to host–pathogen interactions.

The ability to produce medically and agriculturally important secondary metabolites, including many antibiotic, antitumor, herbicidal, and immunosuppressive compounds, is a hallmark of gram-positive actinobacteria belonging to the genus *Streptomyces* (Berdy 2005). These filamentous bacteria are also renowned for their complex morphological life cycle, in which the organism grows as branched vegetative mycelia under conditions of nutrient abundance and then switches to the production of aerial hyphae and spores under conditions of nutrient limitation (Elliot et al. 2008). On the other hand, the capability of *Streptomyces* spp. to cause diseases of economically impor-

tant root and tuber crops is far less appreciated, primarily because only a small number of species possess this attribute. The three best-characterized plant-pathogenic species (*Streptomyces scabies*, *S. turgidiscabies*, and *S. acidiscabies*) cause common scab of potato, a disease that is characterized by round, raised, or pitted corky-like lesions that form on the surface of the tubers. These lesions affect the marketability of the potato crop, leading to significant economic losses in potato-producing regions worldwide (Loria et al. 2006). All three pathogenic streptomycetes are neither tissue nor host specific; therefore, they can also induce scab symptoms on taproot crops such as carrot, radish, and beet, and can infect the fibrous roots of model plants such as *Arabidopsis* and tobacco, leading to root stunting, swelling, and necrosis (Loria et al. 2006). However, infection of the plant host is developmentally constrained in that only expanding plant tissue is susceptible.

A key virulence determinant in scab-causing streptomycetes is a family of phytotoxic secondary metabolites called the thaxtomins, of which thaxtomin A is the most predominant (King and Calhoun 2009). Thaxtomin A is a nitrated 2,5-diketopiperazine synthesized from phenylalanine and 4-nitro-L-tryptophan and is essential for scab disease development (Healy et al. 2000; Johnson et al. 2009; King and Calhoun 2009). It functions by inhibiting cellulose biosynthesis, a conserved process in higher plants, and it also stimulates plant cell H⁺ and Ca²⁺ ion influx and induces programmed cell death (Loria et al. 2006). The biosynthesis of thaxtomin A requires the nonribosomal peptide synthetases TxtA and TxtB as well as a nitric oxide synthase (NOS/TxtD) and a cytochrome P450 monooxygenase, TxtC (Loria et al. 2008). The corresponding biosynthetic genes are highly conserved in *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies* and have not been found in any non-plant-pathogenic *Streptomyces* spp. A regulatory gene, *txtR*, is embedded within the thaxtomin biosynthetic cluster and activates expression of the biosynthetic genes in response to cellobiose (Joshi et al. 2007b). Cellobiose and cellobiose, the smallest subunits of cellulose, both induce thaxtomin A production (Johnson et al. 2007) and may serve as a signal for the presence of expanding plant tissue, which is the site of action of thaxtomin A (Fry and Loria 2002; Scheible et al. 2003).

An additional virulence determinant that has been described in plant-pathogenic streptomycetes is a secreted necrogenic protein called Nec1. Nec1 has no homologues in the protein databases, is required for colonization of the plant host, and may function to suppress plant defense responses (Bukhalid and Loria 1997; Joshi et al. 2007a). Furthermore, a putative virulence gene, *tomA*, is conserved in the three pathogenic

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streptomycetes (Kers et al. 2005) and is homologous to saponinase-encoding genes, which are important for host–pathogen interactions in some plant-pathogenic fungi (Seipke and Loria 2008). Although a *tomA* deletion mutant did not show a reduced virulence phenotype compared with wild-type (WT) *S. scabiei*, the conservation of this gene in pathogenic streptomycetes suggests a role for this gene in plant–microbe interactions (Seipke and Loria 2008). The *nec1*, *tomA*, and thaxtomin biosynthetic genes are all present on a large, mobilizable pathogenicity island in *S. turgidiscabiei* Car8, and the transfer of this island to recipient species is believed to be the mechanism in which new pathogenic *Streptomyces* spp. emerge in agricultural settings (Kers et al. 2005).

Recently, the *S. scabiei* 87-22 genome sequence was completed, and analysis of the sequence revealed a number of additional genes and gene clusters that could potentially be involved in host–pathogen interactions. Here, we describe the identification and characterization of a newly discovered secondary metabolite biosynthetic cluster that is predicted to synthesize a compound similar to coronafacic acid (CFA), a component of the coronatine (COR) phytotoxin produced by several pathovars of the plant pathogen *Pseudomonas syringae*. COR is required for the full virulence phenotype of *P. syringae* (Bender et al. 1999a and b) and a putative *cfa* biosynthetic cluster in a second plant pathogen, *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*), is important for the pathogenic phenotype of that organism (Bell et al. 2004). Through expression analyses and mutational studies, we demonstrate here that the *S. scabiei* CFA-like molecule also plays a role in plant–microbe interactions during the infection process.

RESULTS

Identification of a putative virulence-associated secondary metabolite biosynthetic cluster in *S. scabiei*.

While analyzing the *S. scabiei* 87-22 genome, we identified a cluster of genes that appeared to encode proteins for the synthesis of a polyketide-like secondary metabolite. The cluster is predicted to be approximately 31 kb in size and consists of 15 coding sequences based on gene organization, homology searches and transcriptional analyses (Fig. 1A; Table 1). Thirteen of these genes are oriented in the same direction and many of them are predicted to overlap, which suggests that they are translationally coupled. Analysis of the published genome sequences for the nonpathogenic streptomycetes *S. coelicolor* (Bentley et al. 2002), *S. avermitilis* (Ikeda et al. 2003), and *S. griseus* (Ohnishi et al. 2008), as well as the unpublished genome sequences (available in the public database) for five additional nonpathogenic *Streptomyces* spp. (*S. svaceus*, *S. clavuligerus*, *S. pristinaespiralis*, *Streptomyces* sp. strain Mg1, and *Streptomyces* sp. strain SPB74), indicated that this *S. scabiei* putative secondary metabolite cluster is not conserved in any of these species. However, the cluster is highly similar in structure and organization to the *cfa* biosynthetic cluster found in several pathovars of the plant-pathogenic bacterium *P. syringae*, as well as a putative *cfa* biosynthetic cluster recently identified in another plant pathogen, *Pectobacterium atrosepticum* (Fig. 1B and C). In *P. syringae*, CFA is an intermediate in the biosynthesis of the COR phytotoxin (Fig. 1D), which is an important virulence determinant in that organism (Bender et al. 1999a and b), and mutational studies demonstrated that the putative *cfa* biosynthetic cluster contributes to virulence in *Pectobacterium atrosepticum* (Bell et al. 2004). Given the conservation of this biosynthetic cluster in plant-pathogenic bacteria, together with the published mutational studies in *P.*

syringae and *Pectobacterium atrosepticum*, we hypothesized that the *S. scabiei* biosynthetic cluster is also important for plant–microbe interactions and represents a newly discovered virulence determinant in that organism.

Bioinformatics analyses predict that the *S. scabiei* secondary metabolite biosynthetic cluster produces a novel CFA-like molecule.

Nine of the genes from the *S. scabiei* biosynthetic cluster encode proteins that are homologous to those encoded in the *cfa* clusters from *P. syringae* pv. *tomato* and *Pectobacterium atrosepticum* (Table 1). The *cfa1*-5 genes encode enzymes believed to be responsible for synthesis of the CFA intermediate, 2-carboxy-2-cyclopentenone (CPC), from α -ketoglutarate and malonyl-CoA (Rangaswamy et al. 1998). The *cfa6* and *cfa7* genes encode large multifunctional type-I polyketide synthase (PKS) proteins required for synthesis of the CFA backbone (Table 1). Phylogenetic analysis indicated that the acyltransferase (AT) domains (AT0, AT1, and AT2) from the *S. scabiei* Cfa6 and Cfa7 proteins are most closely related to the corresponding domains from the *P. syringae* and *Pectobacterium atrosepticum* Cfa6 and Cfa7 proteins (Supplementary Fig. S1); because AT domains specify the starter and extender units in polyketide biosynthesis (Katz 2009), this suggests that the same starter and extender units are used in all three organisms. Interestingly, the enoyl reductase (ER2) domain in the *S. scabiei* Cfa7 protein is not present in the corresponding proteins in *P. syringae* pv. *tomato* and *Pectobacterium atrosepticum* (Fig. 2A). Analysis of the domain sequence revealed the presence of the conserved NADP(H) binding motif LXHX(G/A)XGGVG, which is characteristic of these domains (Donadio and Katz 1992). This suggests that the ER2 domain in the *S. scabiei* Cfa7 protein is active and that a double bond that is normally present in the *P. syringae* CFA molecule would be reduced in the *S. scabiei* product (Fig. 2B).

All three biosynthetic clusters contain a *cfl* (coronafacate ligase) gene that is located at the beginning of the *cfa* cluster in *P. syringae* and *Pectobacterium atrosepticum* (Fig. 1B and C) and is present towards the end of the *S. scabiei* biosynthetic cluster (Fig. 1A). Cfl in *P. syringae* is thought to be required for ligation of CFA to coronamic acid (CMA), an ethylcyclopropyl amino acid derived from isoleucine, to give the COR phytotoxin (Fig. 1D) (Bender et al. 1999b). Interestingly, *S. scabiei* is not predicted to synthesize COR because the biosynthetic genes for CMA are not present in the genome; a similar situation has been reported for *Pectobacterium atrosepticum* (Bell et al. 2004). However, the substrate specificity of the *P. syringae* Cfl protein is believed to be relaxed because a variety of coronafacyl-amino acid conjugates have been isolated, including CFA-Ile, CFA-Val, CFA-Ser, and CFA-Thr (Bender et al. 1999b). Therefore, it is conceivable that the Cfl proteins in *Pectobacterium atrosepticum* and *S. scabiei* might generate CFA-amino acid conjugates similar to those observed in *P. syringae*. Alternatively (or in addition), the Cfl orthologues may play a role in polyketide biosynthesis as reported for the *P. syringae* Cfl (Rangaswamy et al. 1997).

Six genes were identified in the *S. scabiei* biosynthetic cluster that have no homologues in the *P. syringae* and *Pectobacterium atrosepticum* *cfa* clusters (Fig. 1A, B, and C). One gene, *scab797II*, encodes a putative 3-hydroxybutyryl-CoA dehydrogenase (Table 1) that might function to reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA, an intermediate in crotonyl-CoA biosynthesis (Chan et al. 2009). Crotonyl-CoA, in turn, is a substrate for the crotonyl-CoA carboxylase/reductase (CCR) enzyme (encoded by *cfa8*) (Table 1) that catalyzes the direct conversion of crotonyl-CoA to ethylmalonyl-CoA (Chan et al. 2009). Genes for both CCR and 3-hydroxybutyryl-CoA dehy-

drogenase have been identified in the concanamycin A, elaiophyllin, indanomycin, and lasalocid antibiotic biosynthetic clusters; the inclusion of such genes in secondary metabolite biosynthetic clusters may serve as a strategy for ensuring that sufficient ethylmalonyl-CoA is available for polyketide biosynthesis (Smith et al. 2008; Chan et al. 2009). Another gene, *scab79691*, encodes a putative P450 monooxygenase that might play a role in post-PKS tailoring of the polyketide product. *Scab79691* contains the conserved heme-binding cysteine residue as well as the EXXR motif, which forms a charge pair in the K-helix of P450s (Zhao and Waterman 2007). Two other genes, *scab79681* and *scab79721*, encoding a putative oxidoreductase and a short-

chain dehydrogenase/reductase, respectively, might also play a role in modification of the *S. scabies* polyketide molecule.

The *scab79591* gene (Fig. 1A; Table 1) encodes a 266-amino-acid protein that shows highest similarity to members of a novel family of *Streptomyces* transcriptional regulators that are typically associated with antibiotic biosynthetic clusters. Members of this family are characterized by the presence of an N-terminal PAS fold-like motif and a C-terminal DNA-binding domain of the LuxR family. The PAS fold is found in cytoplasmic proteins in archaea, bacteria, and eukarya; it has a conserved structure despite having low sequence conservation across different family members (Hefti et al. 2004); and it

Fig. 1. Organization of **A**, the *cfa*-like biosynthetic gene cluster from *Streptomyces scabies* 87-22; **B**, the *cfa* biosynthetic cluster from *Pseudomonas syringae* pv. *tomato* DC3000; and **C**, the putative *cfa* biosynthetic cluster from *Pectobacterium atrosepticum* SCRI1043. Gray block arrows in all three clusters represent known or predicted biosynthetic genes, the black arrow in the *P. atrosepticum* cluster depicts a predicted regulatory gene, and the white arrows represent genes that are unique to the *S. scabies* cluster. The scale bar (2 kb) applies to A, B, and C. **D**, Structure of the *Pseudomonas syringae* coronatine (COR) phytoxin consisting of coronafacic acid (CFA) linked to coronamic acid (CMA).

functions as a sensor of oxygen, redox potential, cellular energy levels, light, or small ligands (Taylor and Zhulin 1999). Immediately downstream of *scab79591* is a gene, *scab79581* (Fig. 1A; Table 1), which encodes a 637-amino-acid protein showing low sequence similarity with other proteins in the database. BlastP analysis indicated that it is 25% identical and 39% similar to a UBA/ThiF-type NAD/FAD-binding fold protein from *Nitrosospora multififormis* (Table 1). The function of *Scab79581* in the *S. scabies* biosynthetic cluster is not known.

Taken together, the in silico analyses indicate that the *S. scabies* polyketide biosynthetic cluster likely produces a compound that is structurally similar but not identical to CFA from *P. syringae*, and that the compound is likely novel in structure.

The type-I PKS genes from the *cfa*-like cluster are conserved in strains of *S. scabies* but are absent from *S. turgidiscabies* and *S. acidiscabies*.

The known virulence genes for thaxtomin A biosynthesis and for Nec1 as well as the putative virulence gene, *tomA*, are all highly conserved among strains of *S. scabies*, *S. acidiscabies*, and *S. turgidiscabies*. It was conceivable that the *S. scabies* polyketide biosynthetic cluster (herein referred to as the *cfa*-like cluster) might also be conserved among these pathogenic species; therefore, Southern analysis was performed to detect the *cfa6* and *cfa7* type-I PKS genes in each of the species. The strains for each species that were analyzed for the *cfa*-like cluster are listed in Table 2; all, with the exception of *S. scabies* 96-16 and *S. acidiscabies* 98-48, were deemed to be pathogenic using a radish seedling bioassay that mainly detects thaxtomin A production (Table 2). Surprisingly, none of the *S. acidiscabies* or *S. turgidiscabies* genomic DNA samples hybridized with either the *cfa6*- or *cfa7*-specific probes (Table 2). On the other hand, both probes hybridized with DNA samples from most of the *S. scabies* strains, which were isolated from a variety of different geographical locations (Table 2). Interestingly, those *S. scabies* strains that were found to contain both *cfa6* and *cfa7* all belong to group 1 *S. scabies* whereas those that did not (e.g., strains 89-04, 96-01, and 96-08) belong to group 2 *S. scabies*. These two groups differ by a single nucleotide in the γ -variable region of the 16S ribosomal DNA gene (Bukhalid et al. 2002).

The *S. scabies cfa*-like cluster is expressed as two messenger RNA transcripts under thaxtomin-inducing conditions.

In light of the fact that newly discovered natural product biosynthetic clusters can be transcriptionally silent in the host organism under laboratory growth conditions (Challis 2008), we wanted to determine whether the *cfa*-like biosynthetic genes are expressed in *S. scabies*. RNA was isolated from *S. scabies* 87-22 mycelia harvested from trypticase soy broth (TSB), oat bran broth (OBB), and thaxtomin-defined medium with cellobiose (TDM-C) liquid cultures and was subjected to reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. Transcription of the *cfa*-like cluster was not detected in the TSB cultures but expression did occur when *S. scabies* was grown in OBB and TDM-C (not shown); these latter two media also support the production of thaxtomin A (Johnson et al. 2007). We further characterized the expression of the *cfa*-like cluster by performing RT-PCR to evaluate whether the genes in the cluster are co-transcribed. In total, 10 products were amplified from cDNA template using PCR primers that were designed to amplify the junction regions between at least two genes in the cluster; no such products were detected when no RT (NRT) control reactions were performed (Fig. 3A and B). This indicates that the *cfa1-scab79721* genes are expressed as a single approximately 27-kb transcriptional unit (Fig. 3A). A second approximately 3-kb transcription unit was also identified that consists of the putative regulatory gene, *scab79591*, as well as the *scab79581* coding sequence; both of these genes are oriented in the opposite direction to the *cfa1-scab79721* genes (Fig. 3A and B).

Transcription of the *cfa*-like genes occurs during plant-microbe interactions.

We predicted that, if the *cfa*-like biosynthetic cluster is important for pathogenicity, then the genes should be expressed when the pathogen is interacting with the plant host. We utilized a green fluorescent protein (GFP) promoter reporter system in order to test this hypothesis. The promoter region for *cfa1*, which drives expression of the large, approximately 27-kb transcript (Fig. 3A), was cloned upstream of a promoterless

Table 1. Predicted function of proteins encoded by the *Streptomyces scabies* 87-22 *cfa*-like cluster and percent identity and similarity to homologous proteins from *Pseudomonas syringae* pv. *tomato* DC3000 and *Pectobacterium atrosepticum*SCRI1043

<i>S. scabies</i> gene	G+C (%)	Predicted protein product ^a	Predicted function ^b	Homologue, identity/similarity (%)	
				DC3000	SCRI1043
<i>scab79581</i>	74	UBA/THIF-type NAD/FAD binding fold protein	Unknown	...	...
<i>scab79591</i>	71	Transcriptional regulator	Gene regulation	...	...
<i>scab79601; cfa1</i>	60	Acyl carrier protein (ACP)	Biosynthesis of starter unit CPC	Cfa1, 59/73	Cfa1, 61/74
<i>scab79611; cfa2</i>	62	Type-II fatty acid dehydratase (DH)	Biosynthesis of starter unit CPC	Cfa2, 67/81	Cfa2, 67/84
<i>scab79621; cfa3</i>	73	Type-II β -ketoacyl synthase (KS)	Biosynthesis of starter unit CPC	Cfa3, 60/74	Cfa3, 61/74
<i>scab79631; cfa4</i>	71	Unknown	Biosynthesis of starter unit CPC	Cfa4, 53/66	Cfa4, 52/69
<i>scab79641; cfa5</i>	71	Acyl-CoA ligase	Biosynthesis of starter unit CPC	Cfa5, 52/68	Cfa5, 53/68
<i>scab79651; cfa6</i>	75	Type-I PKS loading module (AT0-ACP0) + module 1 (KS1-AT1-DH1-ER1-KR1-ACP1)	Biosynthesis of CFA polyketide backbone	Cfa6, 52/64	Cf6, 54/68
<i>scab79661; cfa7</i>	75	Type-I PKS module 2 (KS2-AT2-DH2-ER2-KR2-ACP2)	Biosynthesis of CFA polyketide backbone	Cfa7, 56/67	Cf7, 52/66
<i>scab79671; cfl</i>	72	Acyl-CoA ligase	Generation of CFA-amino acid conjugates	Cfl, 40/59	Cfl, 36/58
<i>scab79681</i>	70	Oxidoreductase	Modification of CFA backbone	...	...
<i>scab79691</i>	69	Cytochrome P450 monooxygenase	Modification of CFA backbone	...	...
<i>scab79701; cfa8</i>	69	Crotonyl-CoA carboxylase/reductase (CCR)	Biosynthesis of ethylmalonyl-CoA extender unit	Cfa8, 36/52	Cfa8b, 35/52
<i>scab79711</i>	72	Hydroxybutyryl-CoA dehydrogenase	Biosynthesis of ethylmalonyl-CoA extender unit	...	...
<i>scab79721</i>	69	Short chain dehydrogenase/reductase	Modification of CFA backbone	...	...

^a AT, acyltransferase; ER, enoylreductase; KR, ketoreductase; TE, thioesterase.

^b CPC, 2-carboxy-2-cyclopentenone.

egfp gene, and promoter activity was assessed by visualizing fluorescence during colonization of plant roots. We were easily able to detect fluorescing mycelia from the *cfa1* promoter reporter strain when it was colonizing the surface of tobacco seedling roots (Fig. 4). The observed fluorescence was well above any background fluorescence observed with a promoterless *egfp* control strain but was generally not as intense as that observed with the positive control strain where *egfp* expression was driven by the strong, constitutive promoter, *ermEp** (Bibb et al. 1994). Similar results were observed when the GFP reporter strains were colonizing radish and *Arabidopsis* seedling roots (not shown). Furthermore, when the GFP reporter strains were incubated in the plant growth medium without tobacco plant material, only the positive control strain displayed significant fluorescence (not shown). Given that expression of the approximately 27-kb *cfa*-like messenger (m)RNA requires transcription of the *scab79591* regulatory gene (discussed below), these results indicate that the two promoter regions driving expression of the *cfa*-like cluster are both active in planta.

Expression of the *cfa*-like biosynthetic genes is dependent on *Scab79591* and on the *bldA* tRNA.

The location of the *scab79591* regulatory gene next to the *cfa*-like biosynthetic genes (Fig. 1A) suggested that the corresponding protein product may function as a pathway-specific regulator for the biosynthetic cluster. Therefore, we made a knockout (KO) strain of *scab79591* by replacing the coding sequence with a [*hyg+oriT*] disruption cassette. Four mutant iso-

lates were recovered and were each verified by PCR and Southern analysis (data not shown). RT-PCR analysis of two of these mutant isolates, Δ *scab79591*_22-3 (not shown) and Δ *scab79591*_32-2 (Fig. 5A), indicated that expression of *cfa5*, *cfa6*, and *cfl* is significantly reduced in these strains compared with the WT. PCR products for these genes were barely visible after 28 cycles (Fig. 5A) and were slightly more visible when the number of PCR cycles was increased to 30 (not shown). On the other hand, expression of *scab79581*, which is co-transcribed with *scab79591* (Fig. 3A and B), was not affected in the Δ *scab79591* mutants (Fig. 5A). We also examined *cfa* gene expression in a strain where *scab79591* was heterologously overexpressed using the *ermEp** promoter. Overexpression of *scab79591* resulted in enhanced expression of *cfa5*, *cfa6*, and *cfl*, whereas expression of *scab79581* was unaffected (Fig. 5A). These results indicate that *Scab79591* activates expression of the approximately 27-kb transcript containing *cfa1-scab79721* but appears to have no effect on the expression of the approximately 3-kb transcript containing *scab79581* and *scab79591*.

Analysis of the *scab79591* coding sequence indicated that it contains a TTA codon that corresponds to Leu81 in the 266-amino-acid *Scab79591* protein. TTA codons are rare in the GC-rich DNA of *Streptomyces* spp. (Wright and Bibb 1992) and are often found in genes associated with secondary metabolite biosynthetic clusters as well as genes that are involved in morphological development (Chater and Chandra 2008). The efficient translation of this codon often requires the *bldA* tRNA as mutants of *bldA* are typically defective in aerial hyphae formation as well as the ability to produce antibiotics (Lawlor et

Fig. 2. A, Organization of the predicted type-I polyketide synthase domains within the *Pseudomonas syringae* pv. *tomato* Cfa7 protein and the corresponding Cfa7 homologues from *Pectobacterium atrosepticum* and *Streptomyces scabies*. KS, ketosynthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ER, enoylreductase; ACP, acyl carrier protein; TE, thioesterase. The predicted ER domain that is unique to the *S. scabies* homologue is indicated in white. **B**, Structure of coronafacic acid (CFA) from *Pseudomonas syringae* and a predicted structure for the CFA-like compound produced by *S. scabies* 87-22.

al. 1987; Leskiw et al. 1991). RT-PCR analysis demonstrated that expression of the *cfa*-like biosynthetic genes is also significantly reduced in a *S. scabies* $\Delta bldA$ mutant compared with the WT. PCR products for *cfa5*, *cfa6*, and *cfl* were barely visible after 28 cycles (not shown) and were slightly more visible with 30 PCR cycles (Fig. 5B). On the other hand, transcription of neither *scab79581* nor *scab79591* was affected in the *bldA* mutant (Fig. 5B), supporting the notion that *Scab79591* does not activate expression from its own promoter.

The product of the *cfa*-like biosynthetic cluster mimics COR in inducing hypertrophy of potato tissue.

The COR phytotoxin from *P. syringae* is known to induce a variety of symptoms in the plant host, including chlorosis, inhibition of root elongation, and stimulation of ethylene production (Bender et al. 1999b). In addition, COR has been reported to elicit the formation of hypertrophic outgrowths in potato tuber tissue (Sakai et al. 1979), an effect that has been utilized as a semiquantitative bioassay for detecting COR activity (Gnanamanickam et al. 1982; Bender et al. 1989; Volksch et al. 1989). To examine the biological activity of the *S. scabies* CFA-like molecule, we first constructed a KO strain of the *cfa*-like biosynthetic cluster by replacing the *cfa6* type-I PKS gene

(Fig. 1A) with a [*hyg+oriT*] disruption cassette. Gene replacement was performed in both the WT (87-22) strain and in a thaxtomin A-deficient ($\Delta txtA$) strain (Table 3) in order to assess the activity of the CFA-like molecule in both the presence and absence of thaxtomin A. Furthermore, we constructed CFA-like overexpression (OE) strains in both the WT and $\Delta txtA$ backgrounds by overexpressing the *scab79591* regulatory gene from the *ermEp** promoter. Strains (WT, WT/vector control, WT/CFA-like OE, $\Delta cfa6_{12-1}$, $\Delta txtA$, $\Delta txtA$ /vector control, $\Delta txtA$ /CFA-like OE, and $\Delta txtA/\Delta cfa6_1$) were then grown in OBB medium for 7 days and culture supernatants were filter sterilized and tested for hypertrophy activity. Although we did not observe any outgrowths of potato tuber tissue with any of the WT background culture filtrates (due to extensive necrosis of the potato tissue by thaxtomin A) (data not shown), we did see a small amount of outgrowth formation with the $\Delta txtA$ and $\Delta txtA$ /vector control culture filtrates (Fig. 6A and B). The extent of tissue hypertrophy was significantly increased when potato tissue was treated with the $\Delta txtA$ /CFA-like OE sample, whereas no hypertrophic outgrowths were observed with the $\Delta txtA/\Delta cfa6_1$ culture filtrate or with uninoculated OBB medium (Fig. 6A and B). The degree of tissue hypertrophy induced by the *S. scabies* supernatants was significantly less than that ob-

Table 2. *Streptomyces* strains, geographical origin, pathogenicity, and DNA hybridization to probes specific for *cfa6* and *cfa7*

Strain ^a	Isolated from	Pathogenicity ^b	<i>cfa6</i> ^{c,d}	<i>cfa7</i> ^{c,e}
<i>S. scabies</i> strains				
87-22 (WT)	Wisconsin, United States	+	+	+
83-08	Long Island NY, United States	+	+	+
84-34 (ATCC49173 ^T)	Upstate NY, United States	+	+	+
84-70	Maine, United States	+	+	+
84-232	West Virginia, United States	+	+	+
85-08	Maine, United States	+	+	+
85-30	Ohio, United States	+	+	+
87-27	Wisconsin, United States	+	+	+
87-67	Wisconsin, United States	+	+	+
89-04	Alaska, United States	+	–	–
95-17	South Africa	+	+	+
95-18	South Africa	+	+	+
95-22	South Africa	+	+	+
96-01	New Brunswick, Canada	+	–	–
96-04	Nova Scotia, Canada	+	+	+
96-06	Ontario, Canada	+	+	+
96-08	Alberta, Canada	+	–	–
96-15	Germany	+	+	+
96-16	Germany	–	+	+
<i>S. turgidiscabies</i> strains				
94-08 (CAR 8)	Japan	+	–	–
94-10 (Hi-C-16)	Japan	+	–	–
94-16 (S-2)	Japan	+	–	–
94-19 (SS-9)	Japan	+	–	–
94-21 (SS-13)	Japan	+	–	–
94-22 (KO-G-2)	Japan	+	–	–
94-23 (KO-G-6)	Japan	+	–	–
98-01 (SY9113 ^T)	Japan	+	–	–
98-05 (TMH93-6)	Japan	+	–	–
98-15 (91Ki-15)	Japan	+	–	–
98-25 (SMN-2)	Japan	+	–	–
<i>S. acidiscabies</i> strains				
84-45	Maine, United States	+	–	–
84-104	Upstate New York, United States	+	–	–
84-110 (ATCC 49003 ^T)	Maine, United States	+	–	–
84-182	Maine, United States	+	–	–
85-06	West Virginia, United States	+	–	–
90-25	Maine, United States	+	–	–
98-48	Japan	–	–	–
98-49	Japan	+	–	–

^a T = type strain and WT = wild type.

^b Pathogenicity (thaxtomin A production) was assessed using a radish seedling bioassay; + = pathogenic and – = nonpathogenic.

^c Symbols + and – = hybridization and no hybridization, respectively.

^d The *cfa6*-specific probe was generated using the primers DRB209 and DRB210.

^e The *cfa7*-specific probe was generated using the primers DRB363 and DRB364.

served with pure COR (500 ng) (Fig. 6A), which may reflect differences in molecule concentrations used in this assay. Alternatively, this bioassay may not be as sensitive for detecting the activity of the CFA-like molecule due to the predicted structural differences compared with COR.

The *S. scabies* *cfa* mutant shows an altered virulence phenotype compared with the WT.

The role of the *cfa*-like biosynthetic cluster in *S. scabies* virulence was assessed using an in vitro tobacco seedling bioassay. Three separate isolates of the $\Delta cfa6$ mutant strain ($\Delta cfa6$ _8-1, 12-1, and 14-1) were tested in this bioassay. All three isolates produced WT levels of thaxtomin A and secreted WT levels of the Nec1 virulence protein (data not shown). The WT and $\Delta cfa6$ mutant strains all caused severe stunting of shoots and roots compared with the control plants, and chlorosis and eventual death was observed upon extended incubation (data not shown).

However, the WT strain also caused extensive necrosis and swelling of primary and lateral roots, which was most evident at the root tips (Fig. 6C and D). The $\Delta cfa6$ mutant strains, on the other hand, caused considerably less root necrosis and swelling in comparison (Fig. 6C and D). Overexpression of the *cfa*-like biosynthetic cluster in strain 87-22 did not significantly affect the WT phenotype in this bioassay (data not shown).

DISCUSSION

Through expression analyses and mutational studies, we have demonstrated that a novel secondary metabolite biosynthetic cluster identified in the *S. scabies* 87-22 genome sequence contributes to host-pathogen interactions. Bioinformatics analyses of this cluster predicted that it synthesizes a metabolite that is structurally related to the virulence-associated CFA compound from *P. syringae* as well as the putative CFA molecule

Fig. 3. Demonstration of co-transcription of the *Streptomyces scabies* *cfa*-like genes using reverse-transcriptase polymerase chain reaction (RT-PCR). **A**, Schematic diagram showing the location and size of the PCR products (1 to 10) that were amplified using *S. scabies* cDNA as the template. The black arrows represent the two messenger RNA transcripts that were identified in this study. **B**, Agarose gel electrophoresis of PCR products generated using *S. scabies* cDNA as template (RT lane). The cDNA was generated from DNase-treated RNA that was harvested from 48-h thaxtomin-defined medium with cellobiose (TDM-C) cultures. Negative control reactions included replacing the cDNA template with water (Water lane) or with RT reaction mix in which the RT enzyme was omitted (NRT lane). *S. scabies* 87-22 genomic DNA served as the template for positive control reactions (Genomic lane).

Fig. 4. In planta expression analysis of the *Streptomyces scabies* *cfa* cluster using confocal fluorescence microscopy. Tobacco seedlings were infected with the *S. scabies* green fluorescent protein reporter strains 87-22/pIJ8641 (positive control), 87-22/pRLDB43-16 (*cfaI* promoter reporter), and 87-22/pRLDB42-2 (negative control), and colonized tobacco roots were analyzed for fluorescence 4 days post inoculation. Uninoculated plants (Control) were included in the analysis. The fluorescence and transmission micrographs shown were captured in a single focal plane. Scale bars = 75 μ m.

Fig. 5. Reverse-transcriptase polymerase chain reaction (PCR) analysis showing the dependence of *Streptomyces scabies* *cfa*-like gene expression on **A**, Scab79591 and **B**, *bldA*. *S. scabies* strains were grown for 72 h in thaxtomin-defined medium with cellobiose (TDM-C) and RNA was prepared and reverse transcribed to cDNA. PCR reactions were performed using either cDNA or water as the template; products were generated using **A**, 28 cycles or **B**, 30 cycles of PCR. The *murX* gene served as a loading control.

Table 3. Bacterial strains, plasmids, and cosmids used in this study

Strain, plasmid or cosmid	Description ^a	Reference or source
<i>Escherichia coli</i> strains		
DH5α	General cloning host	Gibco-BRL
One Shot Top10	Cloning host for TOPO TA Cloning kit	Invitrogen
ET12567	<i>dam</i> [−] , <i>dcm</i> [−] , <i>hsdS</i> [−] ; nonmethylating host (CmR)	MacNeil et al. 1992
BW25113	Host for Redirect PCR targeting system	Gust et al. 2003
<i>Streptomyces scabies</i> strains		
87-22	<i>S. scabies</i> wild-type strain	Loria et al. 1995
Δ <i>bldA</i>	Global regulatory mutant; 87-22 derivative with a deletion of the <i>bldA</i> tRNA gene (AprR)	This study
Δ <i>txtA</i>	Thaxtomin deficient mutant; 87-22 derivative with a deletion of the <i>txtA</i> gene (AprR)	Johnson et al. 2009
Δ <i>scab79591</i>	CFA-like regulatory mutant; 87-22 derivative with a deletion of the <i>scab79591</i> gene (HygR)	This study
Δ <i>cfa6</i>	CFA-like biosynthetic mutant; 87-22 derivative containing a deletion of the <i>cfa6</i> gene (HygR)	This study
Δ <i>txtA</i> /Δ <i>cfa6</i>	Thaxtomin/CFA-like double mutant; Δ <i>txtA</i> derivative with a deletion of the <i>cfa6</i> gene (AprR, HygR)	This study
Plasmids or cosmids		
pUZ8002	Supplies transfer functions for mobilization of <i>oriT</i> -containing vectors from <i>E. coli</i> to <i>Streptomyces</i> (KnR)	Kieser et al. 2000
pCR2.1TOPO	Phagemid cloning vector for PCR products (AmpR, KnR)	Invitrogen
pIJ8641	<i>ermEp</i> ⁺ - <i>egfp</i> reporter plasmid; integrates into the <i>Streptomyces</i> ϕC31 <i>attB</i> site (AprR)	J. Sun, <i>unpublished data</i>
pRLDB42-2	pIJ8641 derivative; promoterless <i>egfp</i> reporter plasmid (AprR)	This study
pRLDB43-16	pRLDB42-2 derivative; <i>S. scabies</i> <i>cfaI</i> p- <i>egfp</i> reporter plasmid (AprR)	This study
pLST9828	pSET152 derivative containing the <i>ermEp</i> ⁺ promoter; integrates into the <i>Streptomyces</i> ϕC31 <i>attB</i> site (AprR)	Butler et al. 1999
pAU3-45	pSET152 derivative containing the <i>tsr</i> coding sequence + promoter (AprR, ThioR)	Bignell et al. 2005
pRLDB50-1a	pLST9828 derivative containing the <i>tsr</i> coding sequence + promoter (AprR, ThioR)	This study
pRLDB51-1	pRLDB50-1a derivative; <i>scab79591</i> overexpression plasmid (AprR, ThioR)	This study
SuperCos1	Cosmid cloning vector (AmpR, KnR)	Stratagene
Cosmid 158	SuperCos1 derivative containing the <i>S. scabies</i> 87-22 <i>cfa</i> -like locus (AmpR, KnR)	This study
Cosmid 1770	SuperCos1 derivative containing the <i>S. scabies</i> 87-22 <i>cfa</i> -like locus (AmpR, KnR)	This study
Cosmid 2237	SuperCos1 derivative containing the <i>S. scabies</i> 87-22 <i>bldA</i> gene (AmpR, KnR)	This study
pIJ773	Template for REDIRECT PCR targeting system, contains the [<i>aac(3)IV+oriT</i>] disruption cassette (AmpR, AprR)	Gust et al. 2003
pIJ10700	Template for REDIRECT PCR targeting system, contains the [<i>hyg+oriT</i>] disruption cassette (AmpR, HygR)	S. O'Rourke, <i>unpublished data</i>
pACRL2-5	<i>scab79591</i> deletion construct derived from Cosmid 158 (AmpR, KnR, HygR)	This study
pRLDB23-1	<i>bldA</i> deletion construct derived from Cosmid 2237 (AmpR, KnR, AprR)	This study
pRLDB24-1	<i>cfa6</i> deletion construct derived from Cosmid 1770 (AmpR, KnR, HygR)	This study

^a CmR, HygR, KnR, AmpR, AprR, and ThioR = chloramphenicol, hygromycin, kanamycin, ampicillin, apramycin, and thiostrepton resistance, respectively.

Fig. 6. A, Potato tuber disk assay for detection of hypertrophy. Tuber disks were treated with 25 μ l of filter-sterilized supernatant from cultures of *Streptomyces scabies* $\Delta txtA$ (ii), $\Delta txtA$ /vector control (iii), $\Delta txtA$ /CFA-like overexpression (OE) (iv), and $\Delta txtA/\Delta cfa6_1$ (v). Control tuber disks were treated with filter-sterilized oat bran broth medium (i) or with pure coronatine (500 ng) (vi). Arrows indicate areas of hypertrophy caused by the specified treatments. Scale bar = 5 mm. **B,** Close-up images of potato tuber disks showing the hypertrophy caused by the $\Delta txtA$ (ii), $\Delta txtA$ /vector control (iii), and $\Delta txtA$ /CFA-like OE (iv) culture filtrates. Scale bar = 1 mm. **C,** Tobacco root disease symptoms induced by *Streptomyces scabies* infection. Tobacco seedlings (*Nicotiana tabacum* cv. Xanthi) growing on Murashige and Skoog agar medium salts + 2% sucrose medium were infected with wild-type *S. scabies* 87-22 (ii) or $\Delta cfa6_12-1$ (iii) or were mock-infected (i) and were then incubated at $21 \pm 2^\circ\text{C}$ and 12 h of daylight for 6 weeks. Arrows indicate roots which have undergone extensive swelling and necrosis as a result of infection. Scale bar = 10 mm. **D,** Close-up image of a tobacco root from an uninfected plant (i), a 87-22-infected plant (ii), and a $\Delta cfa6_12-1$ -infected plant (iii). Scale bar = 3 mm.

from *Pectobacterium atrosepticum*. Our work here is the first report of a *cfa*-like biosynthetic cluster present in a gram-positive plant pathogen, and the conservation of *cfa* and *cfa*-like biosynthetic clusters in different plant-pathogenic bacteria suggests that CFA and CFA-related compounds may be even more important for host–pathogen interactions than previously realized.

Although the metabolite produced by the *S. scabies* biosynthetic cluster is likely similar to CFA, the in silico analyses of the biosynthetic genes strongly suggest that this metabolite is also novel in structure. For example, the C-C double bond that is present in CFA is likely absent from the *S. scabies* metabolite due to the presence of an ER domain in the Cfa7 PKS that is absent from the corresponding *P. syringae* Cfa7 protein (Fig. 2). Furthermore, the *S. scabies* cluster contains additional genes that could function as tailoring enzymes for the polyketide backbone. It is interesting to note, however, that a homologue of the *P. syringae* Cfl protein is conserved in the *S. scabies* cluster. Cfl is believed to be responsible for ligating CFA to the ethylcyclopropyl amino acid, CMA, to form the COR phytotoxin, and it is also thought to generate other coronafacoyl-amino acid conjugates such as CFA-Ile, CFA-Val, CFA-Ser, and CFA-Thr, and may additionally function in CFA biosynthesis (Bender et al. 1999b). The presence of a Cfl homologue in *S. scabies* suggests that the CFA-like molecule might also be linked to an amino acid component to form compounds similar to coronafacoyl-amino acid conjugates. Because *S. scabies* does not contain the biosynthetic genes for CMA, however, it is unlikely that this organism produces COR, as similarly reported for *Pectobacterium atrosepticum* (Bell et al. 2004).

The *S. scabies* *cfa*-like cluster is expressed during plant–microbe interactions as well as in liquid culture under conditions that induce thaxtomin A production. Furthermore, at least two mRNA transcripts are produced during gene expression, one of which is approximately 27 kb in size and includes most of the coding sequences from the gene cluster. A similar result has been reported for the *P. syringae* *cfa* gene cluster, where all of the biosynthetic genes are expressed as a single large transcript (Liyanage et al. 1995). The smaller, approximately 3-kb transcript identified in *S. scabies* includes a transcriptional regulatory gene, *scab79591*, which functions as a positive activator of the *cfa*-like biosynthetic genes but does not control its own expression. A second gene, *scab79581*, is co-transcribed with *scab79591* and may also play a role in gene regulation, although it is currently unclear what the role of the protein product would be. The *Scab79591* regulatory protein belongs to a novel family of *Streptomyces* transcriptional regulators whose members are all associated with secondary metabolite biosynthetic clusters and contain an N-terminal PAS fold-like domain and a C-terminal LuxR-type DNA-binding domain. Two other members of this family, NysRIV and PimM, which are associated with the antibiotic biosynthetic clusters for nystatin and pimarin, respectively, have also been demonstrated to function as pathway-specific transcriptional activators, and PimM also does not affect the expression of its own gene (Anton et al. 2007; Sekurova et al. 2004). Expression of the *S. scabies* *cfa*-like biosynthetic genes is also dependent on the *bldA* tRNA, which is responsible for translation of the rare UUA codon in *Streptomyces* spp. mRNA. There is evidence that the availability of the *bldA* tRNA is growth-phase dependent in streptomycetes and that *bldA* is important for post-transcriptional regulation of TTA-containing genes (Chater and Chandra 2008). A TTA codon is present in the *scab79591* regulatory sequence and a second TTA codon is also found in the *cfa3* biosynthetic gene; this indicates that production of the CFA-like metabolite is likely dependent on the availability of *bldA* in *S. scabies*.

Although the *S. scabies* CFA-like biosynthetic mutant displayed a reduced virulence phenotype with regard to root disease symptoms (Fig. 6C and D), the strain still caused extensive stunting of roots and shoots as well as chlorosis and eventual death of the tobacco plant host. Furthermore, the genes responsible for synthesis of the polyketide backbone were only found in group 1 *S. scabies* strains and were absent from group 2 strains as well as various strains of *S. turgidiscabies* and *S. acidiscabies*. This suggests that the *cfa*-like biosynthetic cluster is not essential for plant pathogenicity in these organisms. Instead, it appears to contribute to the severity of disease symptoms induced by group 1 *S. scabies*. Although COR is a major virulence factor in *P. syringae*, it also contributes mainly to disease severity and is not required for pathogenicity (Bender et al. 1999b). Similarly, *cfa* mutants of *Pectobacterium atrosepticum* are significantly reduced but not abolished in pathogenicity (Bell et al. 2004). Recently, a cytokinin biosynthetic cluster identified in *S. turgidiscabies* Car8 was shown to contribute to plant–microbe interactions; however, this cluster is not conserved in either *S. scabies* or *S. acidiscabies* (Joshi and Loria 2007; Kers et al. 2005). It is conceivable that pathogenic *Streptomyces* spp. might utilize lineage-specific virulence determinants to help establish an infection of the plant host in addition to those virulence determinants (thaxtomin A, Nec1, and TomA) that are shared by all or most pathogenic streptomycetes (Kers et al. 2005).

The precise role of the CFA-like metabolite in *S. scabies* plant pathogenicity is unclear at this time. The COR phytotoxin of *P. syringae* contributes to increased bacterial populations in planta as well as lesion expansion in several plant hosts, including ryegrass, soybean, tomato, and several crucifers (Bender et al. 1999a). In addition, it was recently demonstrated that COR facilitates entry of the pathogen into host leaves by suppressing the closure of stomata, a response that forms part of the plant basal defense system (Melotto et al. 2006, 2008b). COR is structurally related to the plant hormone jasmonic acid (JA) as well as bioactive JA derivatives such as jasmonoyl-isoleucine (JA-Ile) (Katsir et al. 2008a). JA-Ile and structurally related JA-conjugates (e.g., JA-Val) are important signaling molecules in plant defense responses to herbivores and necrotrophic pathogens (Katsir et al. 2008a). They promote the interaction of the COI1 F-box protein, which determines the substrate specificity of the SCF-type E3 ubiquitin ligase, SCF^{COI1}, with a certain class of jasmonate ZIM-domain (JAZ) repressor proteins; this, in turn, leads to the ubiquitination and subsequent degradation of the JAZ proteins and the induction of JA-responsive genes (Katsir et al. 2008b; Melotto et al. 2008a; Thines et al. 2007). Recently, it was demonstrated that COR functions as a molecular mimic of JA-Ile by promoting COI1-JAZ interactions and inducing JA-dependent gene expression (Katsir et al. 2008b; Melotto et al. 2008a). Activation of JA signaling by COR, in turn, suppresses salicylic acid (SA) signaling, which is important in plant defense against biotrophic pathogens such as *P. syringae* (Glazebrook 2005). Thus, COR functions to manipulate plant hormone signaling in order to enhance the susceptibility of the host to pathogen infection. Because the *S. scabies* CFA-like compound appears to share some biological activity with COR, as deduced by the potato hypertrophy bioassay, it is possible that this compound also functions to suppress plant defense responses during *Streptomyces* spp. infection. Interestingly, studies in our lab suggest that JA-dependent signaling is more important than SA-dependent signaling for host defense against pathogenic streptomycetes (M. Joshi and R. Loria, unpublished data). It is intriguing to speculate that the CFA-like metabolite might enhance pathogen infection by functioning as an antagonist of

JA-dependent signaling rather than as a stimulant like COR. Such an activity might be the result of structural differences between COR and the *S. scabies* molecule. Therefore, purification of the CFA-like metabolite for both structural elucidation and assessment of biological activity is the next logical step in characterizing this molecule and its role in *S. scabies* pathogenicity.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and genetic manipulations.

S. scabies, *S. acidiscabies*, and *S. turgidiscabies* strains used in this study are listed in Tables 2 and 3. *S. scabies* 87-22 (WT strain) and other strains were routinely cultivated on the International *Streptomyces* Project (ISP) medium 2 and medium 4 (BD Biosciences, San Jose, CA, U.S.A.) at 28°C. Where required, the medium was supplemented with apramycin (100 µg/ml), hygromycin B (100 µg/ml), kanamycin (50 µg/ml), thiostrepton (25 µg/ml), or nalidixic acid (50 µg/ml). Spore suspensions of *Streptomyces* strains were prepared from 7- to 10-day-old ISP-4 plates and were quantified by dilution plating onto nutrient agar medium (Kieser et al. 2000). DNA was extracted from *Streptomyces* cultures grown in TSB medium (BD Biosciences) for 24 to 48 h at 28°C using the MasterPure gram-positive DNA purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.) according to the manufacturer's instruction. *Streptomyces* spores (10^6 to 10^8) were pregerminated in 2× YT as described (Kieser et al. 2000) and then grown in TSB, TDM-C, or OBB (Johnson et al. 2007) for RNA extraction and analysis. *Escherichia coli* strains used in this study are listed in Table 3. Strains were grown and maintained as described previously (Sambrook et al. 1989) and plasmid-containing cultures were supplemented with ampicillin (100 µg/ml), kanamycin (50 µg/ml), chloramphenicol (25 µg/ml), hygromycin B (100 µg/ml), or apramycin (100 µg/ml). DNA was manipulated in *E. coli* DH5α using standard procedures (Sambrook et al. 1989) and all cosmids, plasmid vectors, and recombinant plasmids used are listed in Table 3. Sequencing of plasmid and PCR products was performed by the Cornell University Life Sciences Core Laboratories Center. All oligonucleotide primers used for sequencing or PCR are listed in Supplementary Table S1. Intergeneric conjugal transfer of DNA into *Streptomyces* spp. was performed according to the protocol described previously (Kieser et al. 2000) except that the spores were not heat shocked. DNA was transferred from the methylation-deficient *E. coli* ET12567 strain that carried the helper plasmid pUZ8002.

Southern analysis of *Streptomyces* spp. DNA.

Genomic DNA (5 µg) was digested overnight with an appropriate restriction enzyme, then separated by electrophoresis on a 1% agarose gel and transferred overnight to nylon membrane (Whatman Co., Sanford, ME, U.S.A.) under neutral conditions as described (Hopwood et al. 1985). Southern analysis was performed using digoxigenin (DIG)-labeled DNA probes (500 to 600 bp) that were generated by PCR using DIG-dUTP (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to the manufacturer's protocol. Hybridizations were routinely carried out overnight at 50 to 55°C in DIG Easy Hyb solution (Roche), and membranes were washed twice for 15 min each time in solution I (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.1% sodium dodecyl sulfate [SDS]) at 50 to 55°C and then twice for 15 min each time in solution II (0.2× SSC and 0.1% SDS) at 65°C. Immunological detection of the hybridized probe was performed according to the manufacturer's direction (Roche Diagnostics).

RNA isolation and analysis of gene expression.

Streptomyces strains were grown in duplicate in 25 ml of TDM-C, OBB, or TSB at 25 ± 2°C and 150 rpm for 24 to 72 h. RNA was prepared from each culture as described previously (Joshi et al. 2007b), except that Turbo DNA-free DNase (Ambion, Austin, TX, U.S.A.) was used to remove any contaminating genomic DNA in the samples. RT-PCR analysis of *Streptomyces* gene expression was carried out as described (Joshi et al. 2007b) using 2 µg of DNase-treated RNA. NRT control templates (RT reactions without reverse-transcriptase enzyme addition) were included in the analysis to verify the absence of genomic DNA in the RNA samples. Taq DNA polymerase (New England Biolabs, Ipswich, MA, U.S.A.) was used for PCR amplification of cDNA templates, and dimethyl sulfoxide (DMSO) (5%) was included in each reaction mix. *murX* (Joshi et al. 2007b) served as the control gene in these studies.

Analysis of co-transcription of the *S. scabies* *cfa*-like genes.

RNA (2 µg) isolated from 48-h TDM-C cultures was reverse-transcribed as described (Joshi et al. 2007b), and control reactions (NRT) were also performed as described above. PCR was performed using Phusion high-fidelity DNA polymerase (Finnzymes, Inc., Woburn, MA, U.S.A.) according to the manufacturer's protocol. DMSO (5%) was included in all reactions, and control PCR templates included water, NRT reaction mix, and genomic DNA (20 ng). PCR products obtained from cDNA template were cloned into pCR2.1TOPO (Table 3) and were sequenced to verify their identity.

In planta expression analysis of the *S. scabies* *cfa*-like cluster.

This was accomplished using a GFP promoter reporter system. The integrative plasmid pIJ8641, harboring the *egfp* gene expressed from *ermEp** (Bibb et al. 1994), was first digested with *KpnI* to release the *ermEp** fragment. The resulting plasmid ends were religated together to form pRLDB42-2, which contained a promoterless *egfp* gene. Next, the *scab79591-cfa1* intergenic region (1,492 bp) was PCR amplified using Cosmid 1770 as the template and the primers DRB282 and DRB283, and the resulting PCR product was cloned into pCR2.1TOPO and sequenced. The insert was then released from the TOPO vector by digestion with *EcoRI*, the ends were blunted, and the fragment was ligated into *EcoRV*-digested pRLDB42-2. The resulting *cfa1* promoter reporter plasmid (pRLDB43-16) was sequenced to confirm that the insert was present in the correct orientation, and was introduced into *S. scabies* 87-22 by intergeneric conjugation from *E. coli*. In addition, pRLDB42-2 and pIJ8641 were separately conjugated into 87-22 to generate strains that were used as negative and positive controls, respectively, for fluorescence analysis.

Expression of the *cfa1* promoter during plant-microbe interactions was determined using confocal laser scanning microscopy. Tobacco seeds (*Nicotiana tabacum* cv. Xanthi) were surface sterilized in 15% bleach (Clorox) and then incubated for 3 to 5 days at 4°C in the dark in sterile 0.1% phytagar (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.). Seeds were aseptically transferred to Murashige and Skoog agar medium (MS) containing 2% sucrose (Murashige and Skoog 1962) and were incubated at 21 ± 2°C under a 12-h photoperiod for 7 to 8 days. The tobacco seedlings were then transferred to individual wells (two seedlings per well) in six-well plates containing 5 ml of 0.5× MS salts medium (pH 6) lacking organic additives. The medium in each well was inoculated with 10^6 spores (in 50 µl of water) of *S. scabies* GFP reporter strain or was mock inoculated with water. In addition, spores were inoculated into 5 ml of 0.5× MS medium lacking tobacco

plant material. A total of six replicates (12 plants) were set up per treatment. The six-well plates were incubated with slow shaking (75 rpm) at $21 \pm 2^\circ\text{C}$ under a 12-h photoperiod for 7 days, and GFP fluorescence from reporter strains colonizing tobacco seedling roots or in the control wells was analyzed using a Leica TCS SP2 microscope (Leica Microsystems, Wetzlar, Germany) as described (Joshi et al. 2007a). The assay was repeated twice.

Targeted deletion of *S. scabies* 87-22 genes.

Deletion of *scab79591*, *cfa6*, and *bldA* was accomplished using the Redirect PCR targeting system as described previously (Gust et al. 2003). Cosmids 158 (harboring the *scab79591* gene), 1770 (containing the *cfa6* gene), and 2237 (containing the *bldA* gene) were each introduced in *E. coli* BW25113 carrying the arabinose-inducible λ RED expression plasmid, pIJ790. Deletion cassettes ([*hyg+oriT*] for *scab79591* and *cfa6*; [*aac(3)IV+oriT*] for *bldA*) were PCR amplified using pIJ10700 or pIJ773 (Table 3) as the template and using the primers DRB292 and DRB293 for *scab79591*, DRB180 and DRB181 for *cfa6*, and DRB211 and DRB212 for *bldA*. The deletion cassettes were gel purified and then electroporated into *E. coli* cells (containing the appropriate cosmid) that had been induced with arabinose, and mutant cosmids that were isolated were verified by PCR or restriction digestion. The *scab79591* mutant cosmid (pACRL2-5), the *cfa6* mutant cosmid (pRLDB24-1), and the *bldA* mutant cosmid (pRLDB23-1) were then separately conjugated into *S. scabies* 87-22 from *E. coli*, and resulting exconjugants were selected for resistance to hygromycin B (pRLDB24-1 and pACRL2-5) or apramycin (pRLDB23-1) and sensitivity to kanamycin. Verification of the *scab79591*, *cfa6*, and *bldA* deletion strains was by Southern analysis and PCR (data not shown).

Overexpression of *scab79591*.

To overexpress the *scab79591* gene, we first made a derivative of the *Streptomyces* integrative expression vector, pLST9828, so that it could be used in apramycin-resistant strains. The thiostrepton resistant gene (*tsr*) and promoter region from pAU3-45 (Table 3) was PCR amplified using the primers DRB332 and DRB333, and the resulting product was cloned into pCR2.1TOPO and authenticated by sequencing. Next, the primers DRB460 and DRB461 were used to amplify the TOPO-cloned fragment to give an approximately 1,100-bp product flanked with *NheI* sites. The product was digested with *NheI*, gel purified, and then ligated into *NheI*-digested pLST9828 to give pRLDB50-1a. The *scab79591* coding sequence was amplified by PCR using Cosmid 158 as the template and the primers DRB296 and DB297. The resulting product was cloned into pCR2.1TOPO, sequenced, then released by digestion of the TOPO clone with *BamHI* to give a 1,063-bp fragment. After gel purification, the *scab79591*-containing DNA fragment was ligated into *BamHI*-digested pRLDB50-1a to give pRLDB51-1, which was sequenced to confirm the correct orientation of the insert. Both pRLDB50-1a and pRLDB51-1 were then introduced into WT *S. scabies* 87-22 as well as a thaxtomin-deficient mutant, Δ *txtA* (Table 3), via intergeneric conjugation from *E. coli*.

Biological activity of the *S. scabies* CFA-like compound.

The *S. scabies* CFA-like compound was tested for the ability to cause hypertrophy of potato tuber disks. Strains of *S. scabies* were grown for 7 days in the dark at $25 \pm 2^\circ\text{C}$ in 5 to 50 ml of OBB medium, after which the mycelia were pelleted and the supernatants were filter-sterilized using 0.2- μm cellulose acetate filters (VWR). Potato tuber disks (cv. Chippewa) were

prepared as described previously (Loria et al. 1995) and were placed onto moist sterile filter paper in petri dishes. Sterile paper disks (0.6 cm in diameter) were then placed on top of each tuber disk, and culture filtrates (25 μl) were pipetted onto each paper disk. Five tuber disks were used per treatment, and filter-sterilized OBB (25 μl) served as the negative control while pure coronatine (500 ng in water) functioned as a positive control. The petri dishes were then wrapped with parafilm and were incubated at $21 \pm 2^\circ\text{C}$ in the dark for 10 days. The assay was repeated once using different biological replicates of the culture filtrates.

Virulence bioassays.

An in vitro radish seedling bioassay was used to assess thaxtomin A production and virulence by strains of *S. scabies*, *S. turgidiscabies*, and *S. acidiscabies*. Seeds (cv. Burpee White) were surface sterilized in 15% (vol/vol) bleach (Clorox) and then incubated for 30 h at $21 \pm 2^\circ\text{C}$ in the dark in a petri dish containing moistened filter paper. Germinated seeds were placed into wells (13 mm in diameter) that were made in 1.5% agar-water plates. *Streptomyces* strains were grown in TSB for 48 h at 28°C , and then the mycelia were pelleted, washed twice with sterile water, and resuspended in 2 ml of water. Mycelial suspensions (200 μl) were each pipetted into six agar wells containing the radish seed, while control seed were treated with 200 μl of sterile water. The plates were wrapped with parafilm and incubated at $21 \pm 2^\circ\text{C}$ under a 12-h photoperiod for 7 days.

An in vitro tobacco bioassay was performed to assess the virulence phenotype of the Δ *cfa6* mutant. *N. tabacum* cv. Xanthi seeds were surface sterilized as described above and then incubated in 0.1% phytagar for 5 days in the dark at 4°C . The seeds were aseptically transferred to MS agar medium (pH 5.8) containing 2% sucrose in Magenta boxes and were incubated at $21 \pm 2^\circ\text{C}$ under a 12-h photoperiod for 15 to 20 days. Spores of *S. scabies* (10^8 to 10^9) in sterile water (1 ml) were used to inoculate each Magenta box in the vicinity of the plant roots while control plants were treated with 1 ml of sterile water. In total, 30 to 40 plants were used per treatment, and disease symptoms were assessed 6 to 8 weeks post inoculation. The assay was repeated twice.

Analysis of thaxtomin A production and secretion of Nec1.

For thaxtomin A production, *Streptomyces* strains were grown in 6×5 ml of OBB in six-well plates for 7 days at $25 \pm 2^\circ\text{C}$ with moderate shaking (approximately 120 rpm). Mycelia were pelleted by centrifugation and were discarded. Thaxtomin A was purified from culture supernatants and was analyzed by high-performance liquid chromatography as described (Johnson et al. 2007). Secretion of Nec1 into culture supernatants was analyzed by growing strains in 25 ml of TSB broth for 24 h and then testing filter-sterilized supernatants in a potato tuber disk assay as described previously (Joshi et al. 2007a).

Bioinformatics analyses.

DNA and protein sequences were routinely analyzed using Vector NTI 10.3.0 (Invitrogen Inc.). Predicted protein functions were assigned based on database similarity searches using BLASTP (National Center for Biotechnology Information). Protein domain analyses were performed using either the Pfam database (Finn et al. 2008) or the SEARCHPKS website in the case of the modular PKS proteins (Yadav et al. 2003). Protein sequence alignments were generated using either AlignX (Vector NTI 10.3.0) or ClustalX (Chenna et al. 2003) and phylogenetic analysis using the maximum likelihood method was performed using PhyML (Guindon and Gascuel 2003).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The National Center for Biotechnology Information genome project database: www.ncbi.nlm.nih.gov/genomes/lproks.cgi
- SEARCHPKS (A Program for Detection and Analysis of Polyketide Synthase Domains) website: www.nii.res.in/searchpk.html
- The Wellcome Trust Sanger Institute *Streptomyces scabies* webpage: www.sanger.ac.uk/Projects/S_scabies